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MUTAGENICITY EVALUATION OF 1-P-NITROPHENYLAZO-2-NAPHTHALENEAMIN--ETC(U)
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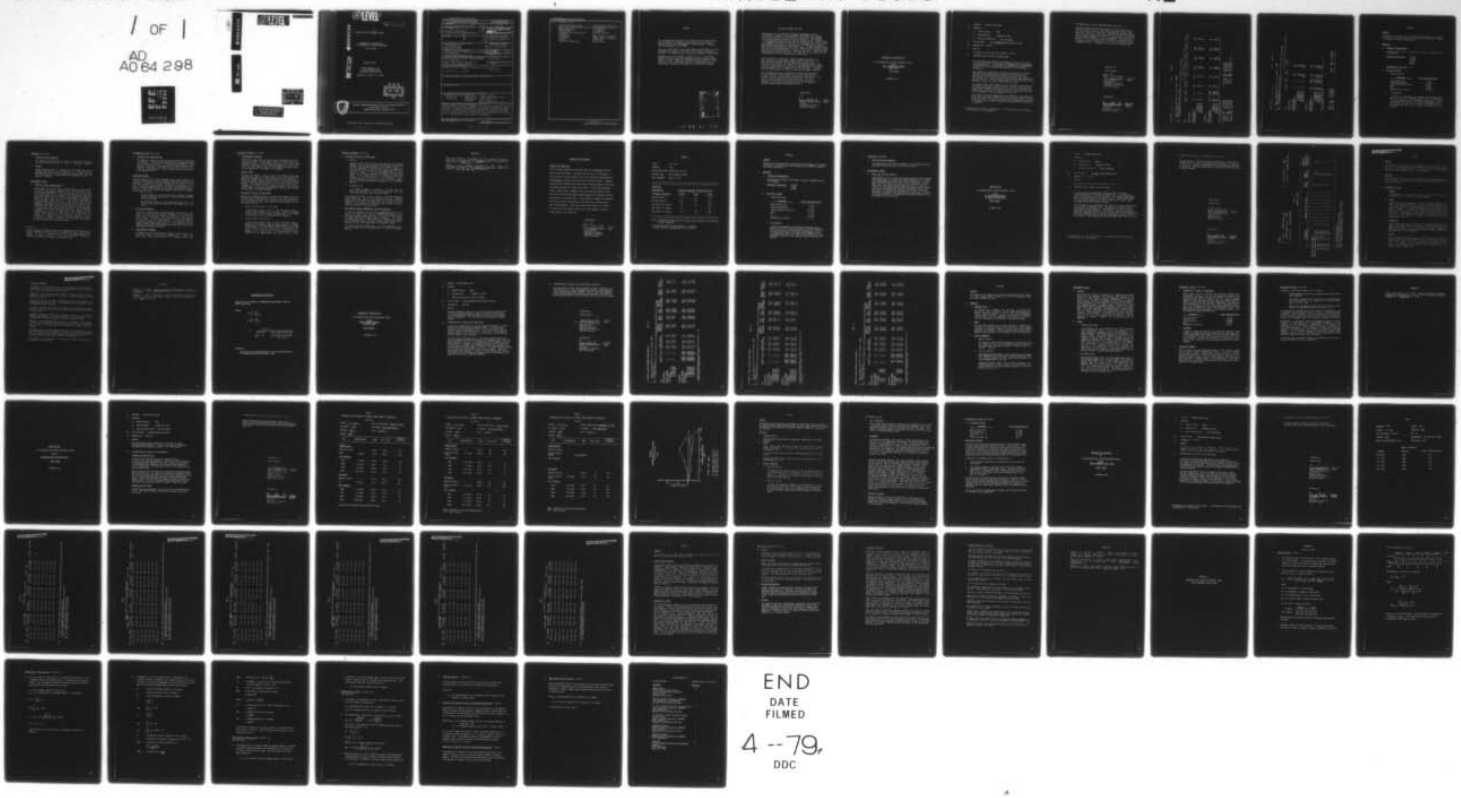

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CONTRACTOR REPORT ARCLS-CR-78058

MUTAGENICITY EVALUATION OF 1-P-NITROPHENYLAZO-2-NAPTHALENEAMINE

FINAL REPORT

DDC FILE COPY

DECEMBER 1978

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20795

CONTRACT NO. DAAK-11-77-C-0106

US ARMY ARMAMENT RESEARCH AND DEVELOPMENT COMMAND
Chemical Systems Laboratory
Aberdeen Proving Ground, Maryland 21010

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MUTAGENICITY
TOXICITY TO BALB/3T3
3-METHYLCHOLANTHRENE CONTROLS
MUTANT CLONES
MUTATION FREQUENCIES
ETHYL METHANESULFONATE CONTROLS (CONT'D)

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

(U) A MUTAGENICITY EVALUATION OF PNNA DYE IS GIVEN BASED ON RESULTS OBTAINED FROM THE AMES SALMONELLA/MICROSOME PLATE TEST, THE IN VITRO TRANSFORMATION OF BALB/3T3 CELLS ASSAY, THE MOUSE LYMPHOMA FORWARD MUTATION ASSAY, THE UNSCHEDULED DNA SYNTHESIS ASSAY AND THE MOUSE DOMINANT LETHAL ASSAY. ALTHOUGH THE DYE IS NOT BELIEVED A SERIOUS GENETIC RISK TO MAMMALS AT LOW CONCENTRATIONS, THE POTENTIAL RISK AS A CARCINOGEN AND MUTAGEN CANNOT BE DISREGARDED.

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19. (U) KEYWORDS - CONT'D

DIMETHYLNITROSAMINE CONTROLS
MOUSE LYMPHOMA FORWARD MUTATION ASSAY
NONACTIVATION ASSAY
MUTATION INDEX
UNSCHEDULED DNA SYNTHESIS ASSAY
THYMIDINE
HYPOXANTHINE
METHOTREXATE
GLYCINE
N-METHYLNITROSOGUANIDINE
MOUSE DOMINANT LETHAL ASSAY
BROMODEOXYURIDINE SENSITIVITY
TK+/-GENOTYPE
TK-/-GENOTYPE
AUTOSOMAL MUTATION
TK
NORMAL FORESKIN FIBROBLASTS
DIPLOID FORESKIN FIBROBLASTS
HYDROXYUREA
BENZO(a)PYRENE

PREFACE

The investigations described in this report were authorized under Contract DAAK-11-77-C-0106, of the same title as this report: The Project/Task was 1M76425D022-04. Work was begun in November 1977 and completed in April 1978.

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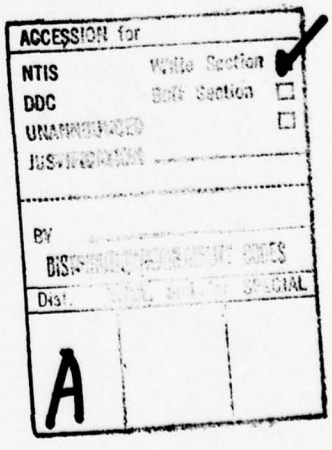


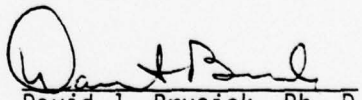
EVALUATION SUMMARY FOR PNNA

PNNA proved to be a mutagen for Salmonella typhimurium inducing predominantly frameshift mutations and for the L5178Y mouse lymphoma cells inducing mutation at the thymidine kinase gene. The response in the mouse lymphoma assay was not strong compared to that obtained in the Salmonella mutants but did meet the requirements for classification as a mutagen. The results in the test for unscheduled DNA synthesis (UDS) with noninduced mouse S9 mix, were negative. The UDS assay was active when Aroclor 1254-induced rat liver S9 was used. The mouse lymphoma assay was active with noninduced mouse liver S9. Another study using dye treated paper also showed weak activity in a bacteria spot test. A mammalian cell transformation assay was negative. This test did not use S9 mix and thus the negative result must be considered inconclusive.

This evaluation, in conclusion, identified genetic potential for PNNA in bacteria, L5178Y mouse lymphoma cells and UDS assay. The fact that this potential was only weakly demonstrated in the mammalian assay and was not expressed in the in vivo dominant lethal test can be interpreted as an indication that PNNA does not represent a serious genetic risk to mammals at low exposure levels. The data could be used, however, to indicate possible carcinogenic potential.

The differences appeared in the areas of potency and requirement for S9 activation. No microsome activation was required for mutagenic activity in the bacteria but appeared to be necessary for activity in the L5178Y cells and AG 1518 cells. This may indicate that the bacteria are metabolizing PNNA to an active mutagen at a high rate and possibly explains the difference in activity between bacteria and mammalian cells.

Submitted by



David J. Brusick, Ph. D. 10/31/7[illegible]
Director Date
Department of Genetics
and Cell Biology

MUTAGENICITY EVALUATION OF

1-P-NITROPHENYLAZO-2-NAPHTHALENEAMINE (PNNA)
IN THE
AMES SALMONELLA/MICROSOME
PLATE TEST

FINAL REPORT

DECEMBER 1978

I. SPONSOR: United States Army

II. MATERIAL*

A. Identification: PNNA

B. Date Received: October 18, 1977

C. Physical Description: Dark red powder

III. TYPE OF ASSAY: Ames Salmonella/Microsome Plate Test

IV. PROTOCOL NO.: DMT-100

V. RESULTS

The results of this assay are presented in Table 1.

VI. INTERPRETATION OF RESULTS AND CONCLUSIONS

The test compound was examined for mutagenic activity in a series of in vitro microbial assays employing Salmonella and Saccharomyces indicator organisms. The compound was tested directly and in the presence of liver microsomal enzyme preparations from Aroclor-induced rats.

The compound was tested over a series of concentrations such that there was either quantitative or qualitative evidence of some chemically-induced physiological effects at the high dose level. The low dose in all cases was below a concentration that demonstrated any toxic effect. The dose range employed for the evaluation of this compound was from 0.1 µg/ml to 1000 µg/ml per plate.

The results of the tests conducted on the compound in the absence of a metabolic system were positive with the strains TA-1538 and TA-98. Dose related increases were obtained over the set of concentrations beginning at 1 µg/plate in Trial #1 and at 0.1 µg/plate in Trial #2. TA-100 responded to the test compound in Trial #2.

The results of the tests conducted on the compound in the presence of a rat liver activation system were positive with the strains TA-1538, TA-98 and TA-100. The activity range for activation tests was similar to the nonactivation results indicating that no metabolic activation is required.

*Information was supplied by the sponsor. If information was not indicated by the sponsor, N.I. was entered.

VI. INTERPRETATION OF RESULTS AND CONCLUSIONS (Continued)

The test compound, PNNA exhibited genetic activity with strains TA-1538, TA-98 and TA-100 in activation and nonactivation assays conducted in this evaluation and is considered as mutagenic under these test conditions. These tests indicate that the test compound does not require metabolic activation to cause genetic activity and that the parent compound in itself is mutagenic. The distinction between Trial #1 and Trial #2 was the amount of histidine added to the overlay tubes. The amount in Trial #2 was the standard amount recommended by Ames. The amount in Trial #1 was slightly lower.

Submitted by:

Study Director

D. R. Jagannath 4-27-78

D. R. Jagannath, Ph.D. Date
Section Chief
Submammalian Genetics
Department of Genetics
and Cell Biology

Reviewed by:

David Brusick 4/27/78

David Brusick, Ph.D. Date
Director
Department of Genetics
and Cell Biology

V. RESULTS — TABLE 1 — TRIAL 1

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: PNNA
B. SOLVENT: DMSO
C. TEST INITIATION DATE: NOV. 29, 1977
NOTE: CONCENTRATIONS ARE GIVEN IN MICROLITERS (UL) OR MICROGRAMS (UG) PER PLATE.

TEST	SPECIES	TISSUE	REVERTANTS PER PLATE											
			TA-1535		TA-1537		TA-1538		TA-98		TA-100		D4*	
			1	2	1	2	1	2	1	2	1	2	1	2
NONACTIVATION														
SOLVENT CONTROL	---	---	20		19		13		24		165		56	
POSITIVE CONTROL**	---	---	478		145		>1000		>1000		840		332	
TEST COMPOUND														
0.100000 UG	---	---	27		9		15		25		135		46	
1.000000 UG	---	---	13		16		89		53		132		49	
10.000000 UG	---	---	27		25		368		131		140		58	
100.000000 UG	---	---	27		17		394		527		150		52	
500.000000 UG	---	---	18		19		305		495		150		68	
ACTIVATION														
SOLVENT CONTROL	RAT	LIVER	28		17		14		45		172		56	
POSITIVE CONTROL***	RAT	LIVER	115		101		427		548		>1000		80	
TEST COMPOUND														
0.100000 UG	RAT	LIVER	29		11		29		34		207		37	
1.000000 UG	RAT	LIVER	20		17		467		446		227		42	
10.000000 UG	RAT	LIVER	29		38		696		797		294		47	
100.000000 UG	RAT	LIVER	80		26		590		603		277		54	
500.000000 UG	RAT	LIVER	27		19		397		424		345		54	

* TRY+ CONVERTANTS PER PLATE

** TA-1535 MNNG 10 UG/PLATE
TA-1537 QM 10 UG/PLATE
TA-1538 NF 100 UG/PLATE
TA-98 NF 100 UG/PLATE
TA-100 MNNG 10 UG/PLATE
D4 MNNG 10 UG/PLATE
SOLVENT DMSO 50 UL/PLATE

*** TA-1535 ANTH 100 UG/PLATE
TA-1537 AMQ 100 UG/PLATE
TA-1538 AAF 100 UG/PLATE
TA-98 AAF 100 UG/PLATE
TA-100 ANTH 100 UG/PLATE
D4 DMNA 100 MICROMOLES/PLATE
SOLVENT DMSO 50 UL/PLATE

- INDICATES TEST WAS NOT DONE

V. RESULTS

TABLE 1 TRIAL 2

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: PNNA
B. SOLVENT: DMSO
C. TEST INITIATION DATE: FEB. 14, 1978
NOTE: CONCENTRATIONS ARE GIVEN IN MICROLITERS (UL) OR MICROGRAMS (UG) PER PLATE.

TEST	SPECIES	TISSUE	REVERTANTS PER PLATE TA-98 1	TA-98 2	TA-100 1	TA-100 2
NONACTIVATION						
SOLVENT CONTROL	---	---	45		577	
POSITIVE CONTROL**	---	---	2325		1694	
TEST COMPOUND						
0.100000 UG	---	---	49		606	
1.000000 UG	---	---	168		578	
10.000000 UG	---	---	592		627	
100.000000 UG	---	---	1415		893	
500.000000 UG	---	---	2232		1651	
1000.000000 UG	---	---	2860		2825	
ACTIVATION						
SOLVENT CONTROL	RAT	LIVER	33		686	
POSITIVE CONTROL***	RAT	LIVER	2962		1800	
TEST COMPOUND						
0.100000 UG	RAT	LIVER	90		759	
1.000000 UG	RAT	LIVER	218		796	
10.000000 UG	RAT	LIVER	2690		1921	
100.000000 UG	RAT	LIVER	3085		1686	
500.000000 UG	RAT	LIVER	3049		2660	
1000.000000 UG	RAT	LIVER	2033		2630	

* TRY+ CONVERTANTS PER PLATE

**

TA-98	NF	10 UG/PLATE
TA-100	EMS	10 UL/PLATE
D4	EMS	10 UL/PLATE
SOLVENT	DMSO	50 UL/PLATE

***

TA-98	ANTH	2.5 UG/PLATE
TA-100	ANTH	2.5 UG/PLATE
SOLVENT	DMSO	50 UL/PLATE

PROTOCOL

1. PURPOSE

The purpose of this study was to evaluate the test material for genetic activity in a microbial assay with and without the addition of mammalian metabolic activation preparations.

2. MATERIALS

A. Indicator Microorganisms

A description of strain verification is given in Standard Operating Procedure.

Salmonella typhimurium	TA-1535
	TA-1537
	TA-1538
	TA-98
	TA-100
Saccharomyces cerevisiae	D4

B. Activation System

1. Reaction mixture

Component	Final Concentration/ml
TPN (sodium salt)	4 µmol
Glucose-6-phosphate	5 µmol
Sodium phosphate (dibasic)	100 µmol
$MgCl_2$	8 µmol
KCl	33 µmol
Homogenate S9 fraction	0.1 ± .05 ml

2. S9 homogenate

A 9,000 x g supernatant was prepared from Sprague-Dawley adult male rat liver induced by Aroclor 1254 five days prior to kill according to the procedure of Ames et al. (1975). S9 samples were coded by lot number and assayed for milligrams protein per milliliter and relative P448/P450 activity by methods described in LBI Technical Data on Rat Liver S9 Product.

2. MATERIALS (Continued)

C. Positive Control Chemicals

The chemicals used for positive controls in the nonactivation and activation assays are given in Table 1 of Section V. Results.

D. Solvent

Either deionized water or dimethylsulfoxide (DMSO) was used to prepare stock solutions of solid materials. All dilutions of test materials were made in either deionized water or DMSO. The solvent employed and its concentration are recorded in Table 1 of Section V. Results.

3. EXPERIMENTAL DESIGN

A. Plate Test (Agar Incorporation)*

Approximately 10^8 cells from an overnight culture of each indicator strain were added to separate test tubes containing 2.0 ml molten agar supplemented with biotin and a trace of histidine. For nonactivation tests, at least 4 dose levels of the test compound were added to the contents of the appropriate tubes and poured over the surfaces of selective agar plates. In activation tests, at least 4 dose levels of the test chemical were added to the appropriate tubes with cells. Just prior to pouring, an aliquot of reaction mixture (0.5 ml containing the 9,000 x g liver homogenate) was added to each of the activation overlay tubes, which were then mixed, and the contents poured over the surface of a minimal agar plate and allowed to solidify. The plates were incubated for 48 hr at 37°C and scored for the number of colonies growing on each plate. D4 yeast plates were incubated at 30°C (nonactivation) and 37°C (activation) for 3-5 days and then scored. The concentrations of all chemicals are given in Table 1 of Section V. Results. Positive and solvent controls using both directly active positive chemicals and those that require metabolic activation were run with each assay.

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* Certain classes of chemicals known to be mutagens and carcinogens do not produce detectable responses using the standard Ames agar incorporation method. Some dialkyl nitrosamines and certain substituted hydrazines are mutagenic in suspension assays, but not in the plate assay. Chemicals of these classes should be screened in a suspension assay.

3. EXPERIMENTAL DESIGN (Continued)

B. Recording and Presenting Data

The numbers of colonies on each plate were counted and recorded on printed forms. These raw data were analyzed in a computer program and reported on a printout. The results are presented as revertants (or convertants for D4) per plate for each indicator strain employed in the assay. The positive and solvent controls are provided as reference points. Other relevant data are provided on the computer printout.

4. EVALUATION CRITERIA

Plate test data consist of direct revertant colony counts obtained from a set of selective agar plates seeded with populations of mutant cells suspended in a semisolid overlay. Because the test chemical and the cells are incubated in the overlay for 2 days, and a few cell divisions occur during the incubation period, the test is semiquantitative in nature. Although these features of the assay reduce the quantitation of results, they provide certain advantages not contained in a quantitative suspension test:

- The small number of cell divisions permits potential mutagens to act on replicating DNA, which is often more sensitive than nonreplicating DNA.
- The combined incubation of the compound and the cells in the overlay permits constant exposure of the indicator cells for 2 days.

A. Surviving Populations

Plate test procedures do not permit exact quantitation of the number of cells surviving chemical treatment. At low concentrations of the test chemical, the surviving population on the treatment plates is essentially the same as that on the negative control plate. At high concentrations, the surviving population is usually reduced by some fraction. Our protocol normally employs several doses ranging over 2 or 3 log concentrations, the highest of these doses being selected to show slight toxicity as determined by subjective criteria.

B. Dose-Response Phenomena

The demonstration of dose-related increases in mutant counts is an important criterion in establishing mutagenicity. A factor that might modify dose-response results for a mutagen would be the

4. EVALUATION CRITERIA (Continued)

B. Dose-Response Phenomena

selection of doses that are too low (usually mutagenicity and toxicity are related). If the highest dose is far lower than a toxic concentration, no increases may be observed over the dose range selected. Conversely, if the lowest dose employed is highly cytotoxic, the test chemical may kill any mutants that are induced, and the compound will not appear to be mutagenic.

C. Control Tests

Positive and negative control assays are conducted with each experiment and consist of direct-acting mutagens for nonactivation assays and mutagens that require metabolic biotransformation in activation assays. Negative controls consist of the test compound solvent in the overlay agar together with the other essential components. The negative control plate for each strain gives a reference point to which the test data are compared. The positive control assay is conducted to demonstrate that the test systems are functional with known mutagens.

D. Evaluation Criteria for Ames Assay

Because the procedures used to evaluate the mutagenicity of the test chemical are semiquantitative, the criteria used to determine positive effects are inherently subjective and are based primarily on a historical data base. Most data sets are evaluated using the following criteria:

1. Strains TA-1535, TA-1537, and TA-1538

   If the solvent control value is within the normal range, a chemical that produces a positive dose response over three concentrations with the lowest increase equal to twice the solvent control value is considered to be mutagenic.

2. Strains TA-98, TA-100, and D4

   If the solvent control value is within the normal range, a chemical that produces a positive dose response over three concentrations with the highest increase equal to twice the solvent control value for TA-100 and 2-3 times the solvent control value for strains TA-98 and D4 is considered to be mutagenic. For these strains, the dose-response increase should start at approximately the solvent control value.

4. EVALUATION CRITERIA (Continued)

D. Evaluation Criteria for Ames Assay

3. Pattern

Because TA-1535 and TA-100 are both derived from the same parental strain (G-46) and because TA-1538 and TA-98 are both derived from the same parental strain (D3052), there is a built-in redundancy in the microbial assay. In general the two strains of a set respond to the same mutagen and such a pattern is sought. It is also anticipated that if a given strain, e.g., TA-1537, responds to a mutagen in nonactivation tests, it will generally do so in activation tests (The converse of this relationship is not expected.). While similar response patterns are not required for all mutagens, they can be used to enhance the reliability of an evaluation decision.

4. Reproducibility

If a chemical produces a response in a single test that cannot be reproduced in one or more additional runs, the initial positive test data lose significance.

The preceding criteria are not absolute, and other extenuating factors may enter into a final evaluation decision. However, these criteria are applied to the majority of situations and are presented to aid those individuals not familiar with this procedure. As the data base is increased, the criteria for evaluation can be more firmly established.

E. Relationship between Mutagenicity and Carcinogenicity

It must be emphasized that the Ames Salmonella/Microsome Plate Test is not a definitive test for chemical carcinogens. It is recognized, however, that correlative and functional relationships have been demonstrated between these two endpoints. The results of comparative tests on 300 chemicals by McCann et al. (1975) show an extremely good correlation between results of microbial mutagenesis tests and in vivo rodent carcinogenesis assays.

All evaluations and interpretation of the data presented in this report are based only on the demonstration, or lack, of mutagenic activity.

REFERENCES

Ames, B.N., McCann, J. and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutation Res. 31, 347-364.

McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975). Detection of carcinogens as mutagens in the Salmonella/microsome test: Assay of 300 chemicals. Proc. Nat. Acad. Sci. 72, 5135-5139.

ADDENDUM TO PNNA REPORT

RESULTS AND CONCLUSION

The tests were conducted on the paper which was impregnated with the PNNA in the plate assays. The paper was cut into 1/2 inch squares and was tested in the presence of rat liver activation system employing the Salmonella indicator strains TA-1538, TA-98 and TA-100. Increased revertants were observed with these strains in the presence of test paper. The average increase was slightly more than 1.5 times the background values in these cases. However, we did not notice clustering of the revertants around the test paper (which happens while testing a positive test material in the spot tests). The increases in number of revertants could have occurred, possible due to the diffusion of the dye into the surrounding media. While these results do not meet our criteria for activity in the standard overlay agar, they appear to indicate a weak effect in this spot test.

Submitted by

D. R. Jagannath 11.6.78

D. R. Jagannath, Ph. D. Date
Section Chief
Submammalian Genetic
Department of Genetics
and Cell Biology

RESULTS

CLIENT: U.S. Army

COMPOUND: PNNA

ACTIVATION SYSTEM: Induced Rat Liver S9

PROTOCOL USED: See Attached Protocol

DATE PERFORMED: June 12, 1978

ACTIVATION CONCENTRATION	INDICATOR ORGANISMS (REVERTANTS/PLATE)		
1/2 Square inch/plate	TA-1538	TA-98	TA-100
Solvent Control*	19	42	210
Positive Control**	846	907	829
Test Paper 1/2" square	36	56	350
Test Paper 1/2" square	37	71	284
Test Paper 1/2" square	27	78	348

* 50 µl Dimethylsulfoxide per plate (used to make dilutions of the positive control compounds).

**TA-1538 and TA-98: 2-Nitrofluorene at 10 µg/plate.
TA-100: N-methylnitrosoguanidine at 2.5 µg/plate.

PROTOCOL

1. PURPOSE

The purpose of this study was to evaluate the test material for genetic activity in a microbial assay with the addition of mammalian metabolic activation preparations.

2. MATERIALS

A. Indicator Microorganisms

A description of strain verification is given in Standard Operating Procedure.

Salmonella typhimurium	TA-1538
	TA-98
	TA-100

B. Activation System

1. Reaction Mixture

Component	Final Concentration/ml
NADP (sodium salt)	4 µmol
Glucose-6-phosphate	5 µmol
Sodium phosphate (dibasic)	100 µmol
$MgCl_2$	8 µmol
KCl	33 µmol
Homogenate S9 fraction	0.1 ml

2. S9 homogenate

A 9,000 x g supernatant was prepared from Sprague-Dawley adult male rat liver induced by Aroclor 1254 five days prior to kill according to the procedure of Ames et al. (1975). S9 samples were coded by lot number and assayed for milligrams protein per milliliter and relative P448/P450 activity by methods described in LBI Technical Data on Rat Liver S9 Product.

2. MATERIALS (Continued)

C. Positive Control Chemicals

The chemicals used for positive controls in the nonactivation and activation assays are given in the results table.

3. EXPERIMENTAL DESIGN

A. Plate Test (Overlay Method)

Approximately 10^8 cells from an overnight culture of each indicator strain were added to separate test tubes containing 2.0 ml molten agar supplemented with biotin and a trace of histidine. The test paper (1/2 inch squares) was placed on the surface of selective agar plates, and then the overlay with cells and 0.5 ml of the 9,000 x g tissue supernatant and required cofactors (core reaction mixture) was spread over the surface of the plate covering the test paper. The plates were incubated for 48 hours at 37°C and scored for the number of colonies growing on each plate. Positive and negative controls using chemicals that require metabolic activation were run with the assay.

EVALUATION OF

1-P-NITROPHENYLAZO-2-NAPHTHALENEAMINE (PNNA)

IN THE
IN VITRO TRANSFORMATION
OF BALB/3T3 CELLS ASSAY

FINAL REPORT

DECEMBER 1978

I. SPONSOR: United States Army

II. MATERIAL*

A. Identification: PNNA

B. Date Received: October 18, 1977

C. Physical Description: Dark red powder

III. TYPE OF ASSAY: In Vitro Transformation Assay

IV. PROTOCOL NO.: DMT-107

V. RESULTS

The results of this assay are presented in Table 1.

VI. INTERPRETATION OF RESULTS AND CONCLUSIONS

The test material was prepared in DMSO and serially diluted in medium to give the final test concentrations. The concentration levels ranged from 0.312 µg/ml to 5 µg/ml. The highest concentration was only slightly toxic to the BALB/3T3 cells. 3-Methylcholanthrene (MCA) was used as the positive control chemical at 5 µg/ml. DMSO was added to the solvent control plates.

The test material was evaluated for its ability to induce morphological transformation in BALB/3T3 cells. The results are shown in Table 1. No increase in the frequency of transformation was obtained at any test level of the dye material. A significant increase in transformation was obtained with the positive control agent 3-Methylcholanthrene All foci scored were Type III and were distributed randomly over all test plates. No dye treated cultures had greater than 1 foci per plate.

*Information was supplied by the sponsor. If information was not indicated by the sponsor, N.I. was entered.

VI. INTERPRETATION OF RESULTS AND CONCLUSIONS (Continued)

The test material, PNNA did not induce morphological transformation in BALB/3T3 cells and was considered not active under the conditions of this assay. Because of the necessity for S9 activation, the lack of response in this study might be due to the lack of bioactivation by microsomal enzymes.

Submitted by:

Study Director

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4/27/78
Date

V. RESULTS

A. Sponsor: United States Army

B. Compound Name or Code: PNNA

C. Description: Dark red powder

D. Solvent Employed: DMSO

E. Test Date: December 30, 1977

TABLE 1

SUMMARY OF DATA

TEST	COMPOUND CONCENTRATION	NUMBER OF PLATES SCORED										TOTAL NUMBER OF FOCI	MEAN NUMBER OF FOCI/PLATE	NUMBER OF FOCI/PLATE CORRECTED FOR SPONTANEOUS
		1	2	3	4	5	6	7	8	9	10			
Negative Control (DMSO)	--	2	3	0	1	1	1	0	1	1	0	10	1.0	--
Positive Control (MCA)	5 µg/ml	6	8	4	7	4	7	6	7	8	7	64	6.4**	5.4
B-1 Dye	0.312 µg/ml	0	0	1	1	2	1	1	2	1	1	10	1.0	--
B-1 Dye	0.625 µg/ml	1	1	0	0	1	1	1	0	1	C	6	0.6	--
B-1 Dye	1.25 µg/ml	0	0	1	1	1	1	0	1	0	0	5	0.5	--
B-1 Dye	2.5 µg/ml	0	1	0	1	1	0	0	1	1	1	6	0.6	--
B-1 Dye	5.0 µg/ml	0	1	1	1	1	0	0	0	1	0	5	0.5	--

3-mc = 3-methylcholanthrene.
C = Plate was contaminated and not scored.
*Data significant from the negative control at $p \leq 0.05$.
**Data significant from the negative control at $p \leq 0.01$.

PROTOCOL

1. PURPOSE

The purpose of this study was to evaluate the test material for an ability to induce malignant transformation of BALB/3T3 cells in vitro. The cells were obtained from Dr. T. Kakunaga. The basic methodology employed is that developed by Dr. T. Kakunaga (1973).

2. MATERIALS

Cells were grown in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal calf serum (FCS). Cultures were then passaged weekly in 60 mm culture dishes.

3. EXPERIMENTAL DESIGN

A. Seeding

Approximately 10^4 cells were seeded into a 25 cm^2 flask and incubated for 24 hr in EMEM to permit attachment.

B. Dosing

After the cells were attached, the control and test chemicals were added to the appropriate plates. Eight to ten replicates per dose level were prepared. 3-methylcholanthrene at 5 µg/ml was used as a positive control, and the test compound solvent was used as the negative control. A minimum of 4 concentrations was tested in the evaluation. The selection of dose levels was based on preliminary cytotoxicity tests measuring the relative cloning efficiency of 3T3 cells and a wide range of chemical concentrations. Chemical exposure was for 72 hr.

C. Incubation

After dosing the cells with the control and test chemicals, the plates were washed free of the compound and replenished with fresh medium containing 5% FCS. The plates were then incubated an additional 3-4 weeks with twice weekly medium changes. Plates were monitored daily for cell integrity.

D. Scoring

After incubation, the medium was aspirated from the cell layer and the cells were washed with buffered saline. The plates were stained with Giemsa, washed, air-dried, and examined for darkly stained foci. All potential foci were examined microscopically. The results are presented as the number of foci per set of replicate plates for each concentration.

4. EVALUATION CRITERIA

The endpoint of carcinogenic activity is determined by the presence of fibroblastic-like colonies which are altered morphologically in comparison to the cells observed in normal cultures.

These cells grow in criss-cross, randomly oriented fashion with overlapping at the periphery of the colony. The colony exhibits dense piling up of cells.

On staining the foci are deeply stained and the cells are basophilic in character and variable in size. These changes are not observed in normal cultures which stain uniformly.

In scoring, smaller foci, which are nearer to the larger foci, are not counted for the reason that they are regarded as having been disseminated from the latter.

Attempts are made to maintain cell cultures with very low or no spontaneous transformation. The data generated at eacn dose level of the test material are analyzed using a t statistic (Brownlee, 1965).

Because of the semiquantitative nature of this assay, dose response curves are not essential for designating a study positive. A significant set of data at any level may be sufficient to indicate a positive response.

The above criteria are only general guidelines for our evaluation. Until the transformation assay becomes a routine test with an extensive historical data base, scientific judgment and expert consultation will still provide the primary input into the final evaluation.

All plates will be retained for 90 days following the assay for inspection or verification.

REFERENCES

Brownlee, K.A. (1965). Statistical Theory and Methodology in Science and Engineering. John Wiley and Sons, New York.

Kakunaga, T. (1973). A quantitative system for assay of malignant transformation by chemical carcinogens using a clone derived from BALB/3T3. Int. J. Cancer 12, 463-473.

CALCULATION OF t-STATISTIC

t-Statistic for Two Means of Independent Random Samples from Two Normal Populations:

Define:

$$\bar{x} = \frac{1}{n_1} \sum_{i=1}^{n_1} x_i$$

$$\bar{y} = \frac{1}{n_2} \sum_{i=1}^{n_2} y_i$$

$$t = \frac{\bar{x} - \bar{y} - d}{\sqrt{\frac{1}{n_1} + \frac{1}{n_2}} \sqrt{\frac{x_i^2 - n_1\bar{x}^2 + y_i^2 - n_2\bar{y}^2}{n_1 + n_2 - 2}}}$$

Reference:

Statistical Theory and Methodology in Science and Engineering, K.A. Brownlee, John Wiley and Sons, 1965.

MUTAGENICITY EVALUATION OF

1-P-NITROPHENYLAZO-2-NAPHTHALENEAMINE (PNNA)

IN THE
MOUSE LYMPHOMA FORWARD
MUTATION ASSAY

FINAL REPORT

DECEMBER 1978

I. SPONSOR: United States Army

II. MATERIAL

A. Identification: PNNA

B. Date Received: October 18, 1977

C. Physical Description: Dark red powder

III. TYPE OF ASSAY: *Mouse Lymphoma Forward Mutation Assay*

IV. PROTOCOL NO.: DMT-106

V. RESULTS

The data presented in Tables 1A, 1B and 1C show the concentrations of the test compound employed, number of mutant clones obtained, surviving populations after the expression period, and calculated mutation frequencies. All calculations are performed by computer program.

VI. INTERPRETATION OF RESULTS AND CONCLUSIONS

A series of concentrations of the test material were made in culture medium from a stock solution prepared in DMSO. Two trials were conducted using uninduced mouse liver S9 mix with concentrations as high as 2 mg/ml of the test material. The toxicity of the compound appeared higher in the first trial than the second. No explanation could be given for the difference.

The test results from both trials of the Mouse Lymphoma Assay with mouse S9 suggested that the dye material was mutagenic under activation test conditions. In Trial 1, an increase in mutant frequency occurred at the dose of 1.67 mg/ml; the frequency was more than 2.5 times the solvent control. However, no dose-response effect was observed, even at other relatively toxic doses. In Trial 2, a similar increase (greater than 2.5-fold over the control) occurred at 0.75 mg/ml. Again, no dose-response was observed. In Trial 3, Aroclor-induced rat liver S9 was employed, and no increase in mutagenic activity was observed that meets our criteria for a positive response.

VI. INTERPRETATION OF RESULTS AND CONCLUSIONS (Continued)

The test material, PNNA, was not mutagenic without activation or in the presence of rat liver S9 metabolic activation. With the mouse liver S9 activation system, however, there is a possible mutagenic effect of PNNA observed in two trials of the assay. The lack of a dose-response nature of the effect may be due to solubility problems.

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10/17/78

David J. Brusick, Ph.D. Date
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and Cell Biology

4a. SUMMARY OF MOUSE LYMPHOMA (L5178Y) RESULTS

TABLE 1A

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: PNNA
B. SOLVENT: DMSO
C. TEST DATE: 12/27/77
NOTE: CONCENTRATIONS ARE GIVEN IN MICROLITERS (UL) OR MICROGRAMS (UG) OR NANOLITERS(NL) PER MILLILITER.

TEST	S-9 SOURCE	S-9 TISSUE	DAILY COUNTS (CELLS/ML X 10E5) 1	2	3	RELATIVE SUSPENSION GROWTH (% OF CONTROL)	TOTAL MUTANT CLONES	TOTAL VIABLE CLONES	RELATIVE CLONING EFFICIENCY (% OF CONTROL)	PERCENT RELATIVE GROWTH*	MUTANT FREQUENCY** (X 10E-6)
NONACTIVATION											
SOLVENT CONTROL	---	---	16.5	17.0	8.3	100.0	32.0	255.0	100.0	100.0	12.5
NEGATIVE CONTROL	---	---	14.0	17.9	7.1	76.4	33.0	280.0	109.8	83.9	11.8
EMS .5UL/ML	---	---	11.2	13.6	6.0	39.3	361.0	122.0	47.8	18.8	295.9
TEST COMPOUND											
0.22000 MG/ML	---	---	8.2	17.6	8.2	50.8	22.0	198.0	77.6	39.5	11.1
0.33000 MG/ML	---	---	8.8	12.4	7.6	35.6	16.0	240.0	94.1	33.5	6.7
0.49000 MG/ML	---	---	8.4	7.6	5.8	15.9	9.0	339.0	132.9	21.1	2.7
0.74000 MG/ML	---	---	6.6	3.6	7.8	6.7	--	306.0	120.0	8.1	--
ACTIVATION											
SOLVENT CONTROL	MOUSE	LIVER	12.0	14.6	7.9	100.0	30.0	297.5	100.0	100.0	10.1
NEGATIVE CONTROL	MOUSE	LIVER	16.2	15.9	8.0	148.9	29.0	255.0	85.7	127.6	11.4
DMN .5UL/ML	MOUSE	LIVER	8.8	11.0	4.4	30.8	172.0	70.0	23.5	7.2	245.7
TEST COMPOUND											
0.22000 MG/ML	MOUSE	LIVER	10.0	10.0	6.6	47.7	11.0	156.0	52.4	25.0	7.1
0.33000 MG/ML	MOUSE	LIVER	7.0	11.8	8.0	47.7	22.0	210.0	70.6	33.7	10.5
0.49000 MG/ML	MOUSE	LIVER	7.2	14.0	5.8	42.2	19.0	219.0	73.6	31.1	8.7
0.74000 MG/ML	MOUSE	LIVER	4.2	11.4	5.0	17.3	18.0	232.0	78.0	13.5	7.8
1.11000 MG/ML	MOUSE	LIVER	4.2	6.4	3.4	6.6	20.0	151.0	50.8	3.4	13.2
1.67000 MG/ML	MOUSE	LIVER	3.8	6.6	2.0	2.9	25.0	91.0	30.6	0.9	27.5

* (RELATIVE SUSPENSION GROWTH X RELATIVE CLONING EFFICIENCY) / 100
** (MUTANT CLONES / VIABLE CLONES) X 10E-6

SUMMARY OF MOUSE LYMPHOMA ASSAY RESULTS

TABLE 1B

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: PNNA
B. SOLVENT: DMSO
C. TEST DATE: 02/26/78
NOTE: CONCENTRATIONS ARE GIVEN IN MICROLITERS (UL) OR MICROGRAMS (UG) OR NANOLITERS(NL) PER MILLILITER.

TEST	S-9 SOURCE	S-9 TISSUE	DAILY COUNTS 1	2	3	RELATIVE SUSPENSION GROWTH (% OF CONTROL)*	TOTAL MUTANT CLONES	TOTAL VIABLE CLONES	RELATIVE CLONING EFFICIENCY (% OF CONTROL)	PERCENT RELATIVE GROWTH*	MUTANT FREQUENCY** X 10E-6
NONACTIVATION											
SOLVENT CONTROL	---	---	10.4	13.3	14.0	100.0	30.0	191.0	100.0	100.0	15.0
NEGATIVE CONTROL	---	---	13.0	14.2	11.2	106.8	40.0	177.0	92.7	98.9	22.6
EMS 0.5UL/ML	---	---	7.8	12.0	9.4	45.4	594.0	81.0	42.4	19.3	733.3
TEST COMPOUND											
0.500000000 MG/ML	---	---	8.6	11.4	13.6	68.9	44.0	195.0	102.1	70.3	22.6
0.750000000 MG/ML	---	---	9.0	8.8	12.4	50.7	48.0	210.0	109.9	55.8	22.9
1.000000000 MG/ML	---	---	8.4	12.8	12.2	67.7	C	241.0	126.2	85.5	C
1.500000000 MG/ML	---	---	11.0	12.6	8.8	63.0	C	250.0	130.9	82.4	C
2.000000000 MG/ML	---	---	10.0	7.4	7.4	28.3	36.0	227.0	118.8	33.6	15.9
ACTIVATION											
SOLVENT CONTROL	MOUSE	LIVER	9.3	14.3	12.8	100.0	38.5	167.0	100.0	100.0	23.0
NEGATIVE CONTROL	MOUSE	LIVER	10.6	13.8	14.2	122.0	C	C			C
DMN 0.5UL/ML	MOUSE	LIVER	5.6	9.0	4.8	14.2	474.0	30.0	18.0	2.6	1580.0
TEST COMPOUND											
0.500000000 MG/ML	MOUSE	LIVER	9.2	15.2	15.0	123.2	39.0	143.0	85.6	105.5	27.3
0.750000000 MG/ML	MOUSE	LIVER	10.0	10.8	10.2	64.7	71.0	110.0	65.9	42.6	64.5
1.000000000 MG/ML	MOUSE	LIVER	9.2	10.2	10.4	57.3	41.0	80.0	47.9	27.5	51.3
1.500000000 MG/ML	MOUSE	LIVER	8.8	15.2	10.2	80.1	C	C			C
2.000000000 MG/ML	MOUSE	LIVER	8.4	13.2	9.8	63.8	C	152.0	91.0	58.1	C

* (RELATIVE SUSPENSION GROWTH X RELATIVE CLONING EFFICIENCY) / 100
** (MUTANT CLONES / VIABLE CLONES) X 10E-6
C = Contamination

TABLE 1C

4. SUMMARY OF MOUSE LYMPHOMA (L5178Y) RESULTS

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: PNNA
B. SOLVENT: DMSO
C. TEST DATE: 04/10/78
NOTE: CONCENTRATIONS ARE GIVEN IN MICROLITERS (UL) OR MICROGRAMS (UG) OR NANOLITERS(NL) PER MILLILITER.

TEST		S-9 SOURCE	S-9 TISSUE	DAILY COUNTS (CELLS/ML X 10E5) 1	2	3	RELATIVE SUSPENSION GROWTH (% OF CONTROL)	TOTAL MUTANT CLONES	TOTAL VIABLE CLONES	RELATIVE CLONING EFFICIENCY (% OF CONTROL)	PERCENT RELATIVE GROWTH*	MUTANT FREQUENCY** X 10E-6
NONACTIVATION												
SOLVENT CONTROL		---	---	6.6	16.6	6.4	100.0	63.0	228.5	100.0	100.0	27.0
NEGATIVE CONTROL		---	---	8.4	13.0	13.6	211.8	70.0	102.0	44.6	94.5	68.6
EMS 0.5UL/ML		---	---	6.6	9.8	11.1	102.4	437.0	110.0	48.1	49.3	397.3
TEST COMPOUND												
0.500	MG/ML	---	---	13.8	9.6	14.2	268.3	44.0	115.0	50.3	135.0	38.3
0.750	MG/ML	---	---	8.8	13.0	14.2	231.7	52.0	87.0	38.1	88.2	59.8
1.000	MG/ML	---	---	9.0	7.8	15.4	154.2	67.0	130.0	56.9	87.7	51.5
1.500	MG/ML	---	---	4.6	25.8	7.2	121.9	61.0	136.0	59.5	72.5	44.9
2.000	MG/ML	---	---	10.0	10.2	13.0	189.1	55.0	136.0	59.5	112.6	40.4
ACTIVATION												
SOLVENT CONTROL		RAT	LIVER	6.9	14.5	17.5	100.0	55.0	153.0	100.0	100.0	35.0
NEGATIVE CONTROL		RAT	LIVER	9.8	15.0	15.6	131.0	62.0	198.0	129.4	169.5	31.3
DMN 0.5UL/ML		RAT	LIVER	6.0	4.4	5.8	8.7	260.0	59.0	38.6	3.4	440.7
TEST COMPOUND												
0.500	MG/ML	RAT	LIVER	8.6	11.6	14.8	84.3	22.0	141.0	92.2	77.7	15.6
0.750	MG/ML	RAT	LIVER	6.4	17.6	7.0	45.0	48.0	133.0	86.9	39.1	36.1
1.000	MG/ML	RAT	LIVER	6.6	19.0	11.0	78.8	62.0	130.0	85.0	66.9	47.7
1.500	MG/ML	RAT	LIVER	9.6	12.0	11.0	72.4	43.0	166.0	108.5	78.5	25.9
2.000	MG/ML	RAT	LIVER	6.4	18.0	11.6	76.3	67.0	124.0	81.0	61.9	54.0

* (RELATIVE SUSPENSION GROWTH X RELATIVE CLONING EFFICIENCY) / 100
** (MUTANT CLONES / VIABLE CLONES) X 10E-4

PROTOCOL

1. PURPOSE

The purpose of this study was to evaluate the test material for specific locus forward mutation induction in the L5178Y thymidine kinase (TK) mouse lymphoma cell assay.

2. MATERIALS

A. Indicator Cells

The Fischer mouse lymphoma cell line used in this study was derived from cell line L5178Y. The cells are heterozygous for a specific autosomal mutation at the TK locus and are bromodeoxyuridine (BUdR) sensitive. Scoring for mutation was based on selecting cells that have undergone forward mutation from a TK+/- to a TK-/- genotype by cloning them in soft agar with BUdR.

B. Media

The cells were maintained in Fischer's medium for leukemic cells of mice with 10% horse serum and sodium pyruvate. Cloning medium consisted of Fischer's medium with 20% horse serum, sodium pyruvate, and 0.37% agar. Selection medium was made from cloning medium by the addition of 7.5 mg BUdR to 100 ml cloning medium.

C. Control Compounds

1. Negative control

The solvent in which the test compound was dissolved was used as a negative control and is designated as solvent control in the data table. The actual solvent is listed in Table 1 of Section V. Results.

2. Positive controls

Ethylmethanesulfonate (EMS), which induces mutation by base-pair substitution, was dissolved in culture medium and used as a positive control for the nonactivation studies at a final concentration of 0.5 µl/ml.

Dimethylnitrosamine (DMN), which requires metabolic biotransformation by microsomal enzymes, was used as a positive control substance for the activation studies at a final concentration of 0.5 µl/ml.

3. EXPERIMENTAL DESIGN

A. Toxicity

The solubility, toxicity, and doses for all chemicals were determined prior to screening. The effect of each chemical on the survival of the indicator cells was determined by exposing the cells to a wide range of chemical concentrations in complete growth medium. Toxicity was measured as loss in growth potential of cells induced by a 4-hr exposure to the chemical followed by a 24-hr expression period in growth medium. A minimum of 4 doses was selected from the concentration range by using as the highest dose a level that showed at least 50% reduction in growth potential. At least 3 lower doses, including levels which were below the toxic range, were added. Those compounds that were relatively nontoxic to cells growing in suspension were tested at concentrations of up to 10 mg/ml when solubility permitted. Toxicity produced by chemical treatment was monitored during the experiment.

B. Assays

1. Nonactivation assay

The procedure used is a modification of that reported by Clive and Spector (1975). Prior to each treatment, cells were cleansed of spontaneous TK-/- by growing them in a medium containing thymidine, hypoxanthine, methotrexate, and glycine (THMG). This medium permits the survival of only those cells that produce TK, and can therefore utilize the exogenous thymidine from the medium. The test compound was added to the cleansed cells in growth medium at the predetermined doses for 4 hr. The mutagenized cells were washed, fed, and allowed to express in growth medium for 3 days. At the end of this expression period, TK-/- mutants were detected by cloning the cells in the selection medium for 10 days. Surviving cell populations were determined by plating diluted aliquots in nonselective growth medium.

2. Activation assay

The activation assay differs from the nonactivation assay in the following manner only. Two milliliters of the reaction mixture were added to 10 ml growth medium containing the desired number of cleansed cells. After adding the test compound, the flask was incubated on a rotary shaker for 4 hr. The incubation period was terminated by washing the cells twice with growth medium. The washed treated cells were then allowed to express for 3 days and were cloned as indicated for the nonactivated cells.

3. EXPERIMENTAL DESIGN (Continued)

C. Preparation of 9,000 x g Supernatant

Male, random bred mice (HA/ICR) were killed by cranial blow, decapitated, and bled. The liver was immediately dissected from the animal using aseptic technique and placed in ice-cold 0.25 M sucrose buffered with Tris buffer at pH 7.4. When an adequate number of livers had been collected, they were washed twice with fresh buffered sucrose and completely homogenized. The homogenate was centrifuged for 20 min at 9,000 x g in a refrigerated centrifuge. The supernatant from this centrifuged sample was retained and frozen at -80°C until used in the activation system. This microsome preparation was added to a core reaction mixture to form the activation system described below:

Component	Final Concentration/ml
TPN (sodium salt)	6 µmol
Isocitric acid	35 µmol
Tris buffer, pH 7.4	28 µmol
$MgCl_2$	2 µmol
Homogenate S9 fraction	100 µl

D. Screening

A mutation index was derived by dividing the number of clones formed in the BUdR-containing selection medium by the number found in the same medium without BUdR. The ratio was then compared to that obtained from other dose levels and from positive and negative controls. Colonies were counted on an electronic colony counter that resolves all colonies greater than 200 microns in diameter.

4. EVALUATION CRITERIA

Several criteria have been established which, if met, provide a basis for declaring a material genetically active in the Mouse Lymphoma Forward Mutation Assay. These criteria are derived from a historical data base and are helpful in maintaining uniformity in evaluations from material to material, and run to run. While these criteria are reasonably objective, a certain amount of flexibility may be required in making the final evaluation since absolute criteria may not be applicable to all biological data.

4. EVALUATION CRITERIA (Continued)

A compound is considered mutagenic in this assay if:

- A dose-response relationship is observed over 3 of the 4 dose levels employed.
- The minimum increase at the high level of the dose-response curve is at least 2.5 times greater than the solvent and/or negative control values.
- The solvent and negative control data are within the normal range of the spontaneous background for the TK locus.

All evaluations of mutagenic activity are based on consideration of the concurrent solvent and negative control values run with the experiment in question. Positive control values are not used as reference points, but are included to ensure that the current cell population responds to direct and promutagens under the appropriate treatment conditions.

Occasionally, a single point within a concentration range will show an increase 2.5 times greater than the spontaneous background. If the increase is at the high dose, is reproducible, and if an additional higher dose level is not feasible because of toxicity, the chemical can be considered mutagenic. If the increase is internal within the dose range and is not reproducible, the increase will normally be considered aberrant. If the internal increase is reproducible, several doses clustered around the positive concentration will be examined to either confirm or reject the reliability of the effect.

As the data base on the assay increases, the evaluation criteria can be expected to become more firmly established.

REFERENCES

Clive, D. and Spector, J.F.S. (1975). Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. Mutation Res. 31, 17-29.

EVALUATION OF

1-P-NITROPHENYLAZO-2-NAPHTHALENEAMINE (PPNA)

IN THE

UNSCHEDULED DNA SYNTHESIS ASSAY

FINAL REPORT

DECEMBER 1978

I. SPONSOR: United States Army

II. MATERIAL*

A. Identification: PNNA

B. Date Received: October 18, 1977

C. Physical Description: Dark red powder

III. TYPE OF ASSAY: Unscheduled DNA Synthesis

IV. PROTOCOL NO.: DMT-108

V. RESULTS

The test material was evaluated for its ability to induce unscheduled DNA synthesis in normal human foreskin fibroblasts. The results are presented in Tables 1, 2, 3 and Figure 1.

VI. INTERPRETATION OF RESULTS AND CONCLUSIONS

Studies with Mouse S9 Mix

A toxicity test covering a series of concentrations of 5 µg/ml to 100 µg/ml was conducted. Based on the lack of toxicity this dose range was employed in the test. Under test conditions, it was observed that substantial toxic effects were obtained in the activation assay. A second test (Table 2) was conducted using lower concentrations.

The test material did not induce any indication of compound-related UDS in either study. The levels of ^{3}H-thymidine incorporation were near 100% of the controls over the first two test concentrations and the two highest concentrations resulted in significantly lower uptake. This may be attributed to compound induced inactivation of repair enzymes. The level of response from Benz(α)pyrene was not significant as a positive control at either 1 µg/ml or 10 µg/ml.

Studies with Rat S9 Mix

A third study was conducted (S9 activation only) using PCB-induced rat S9. The results showed a dose-related increase significant at 5 µg/ml.

VI. INTERPRETATION OF RESULTS AND CONCLUSIONS (Continued)

Based on these data, the test material, PNNA was not active in the Unscheduled DNA Synthesis Assay with noninduced mouse tissue but did show significant UDS with PCB-induced rat liver.

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4/27/78
Date

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Director
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4/27/78
Date

TABLE 1

UNSCHEDULED DNA SYNTHESIS IN NORMAL HUMAN FORESKIN FIBROBLASTS

AG 1518

CLIENT: U. S. Army

DATE OF INITIATION: March 14, 1978

LBI ASSAY # : 2414

S9 SOURCE: Noninduced Mouse

CHEMICAL: PNNA

CELLS: AG 1518

SOLVENT: DMSO

TEST	CONCENTRATION	µgDNA	DPM/µg DNA	PERCENT OF CONTROL
Trial Nonactivation				
Negative Control	--	12.87	115.5	100
MNNG	5 µg/ml	18.15	406.0	261*
Test Compound				
PNNA	5 µg/ml	18.15	102.0	88
PNNA	10 µg/ml	25.91	112.4	97
PNNA	50 µg/ml	20.30	55.8	48
PNNA	100 µg/ml	22.44	52.5	45
Activation				
Negative Control	--	13.70	191.9	100
BαP	10 µg/ml	16.17	193.9	101
Test Compound				
PNNA	5 µg/ml	19.80	145.4	76
PNNA	10 µg/ml	21.78	171.6	89
PNNA	50 µg/ml	16.50	34.3	18
PNNA	100 µg/ml	20.96	48.8	25

*Significantly different from the control value.

TABLE 2

UNSCHEDULED DNA SYNTHESIS IN NORMAL HUMAN FORESKIN FIBROBLASTS

AG 1518

CLIENT: U. S. Army DATE OF INITIATION: July 5, 1978

LBI ASSAY #: 2414 S9 SOURCE: Noninduced Mouse

CHEMICAL: PNNA CELLS: AG 1518

SOLVENT: DMSO

TEST	CONCENTRATION	µgDNA	DPM/µg DNA	PERCENT OF CONTROL
NONACTIVATION				
Negative Control	--	24.75	46	100
Positive Control (MNNG)	5.0 µg/ml	25.58	123	267
TEST COMPOUND:				
PNNA	0.5 µg/ml	17.30	35	76
PNNA	1.0 µg/ml	22.28	28	61
PNNA	5.0 µg/ml	22.11	34	74
PNNA	10.0 µg/ml	24.42	20	44
ACTIVATION				
Negative Control	--	30.86	50	100
Positive Control (BαP)	1.0 µg/ml	29.04	54	108
TEST COMPOUND:				
PNNA	0.5 µg/ml	31.52	39	78
PNNA	1.0 µg/ml	22.28	42	84
PNNA	5.0 µg/ml	41.25	37	74
PNNA	10.0 µg/ml	21.94	34	68

MNNG = N-methyl-N'-nitro-N-nitrosoguanidine
BαP = Benz( )pyrene

TABLE 3

UNSCHEDULED DNA SYNTHESIS IN NORMAL HUMAN FORESKIN FIBROBLASTS

AG 1518

CLIENT: U. S. Army

DATE OF INITIATION: September 14, 1978

LBI ASSAY #: 2414

S9 SOURCE: PCB-induced Rat

CHEMICAL: PNNA

CELLS: AG 1518

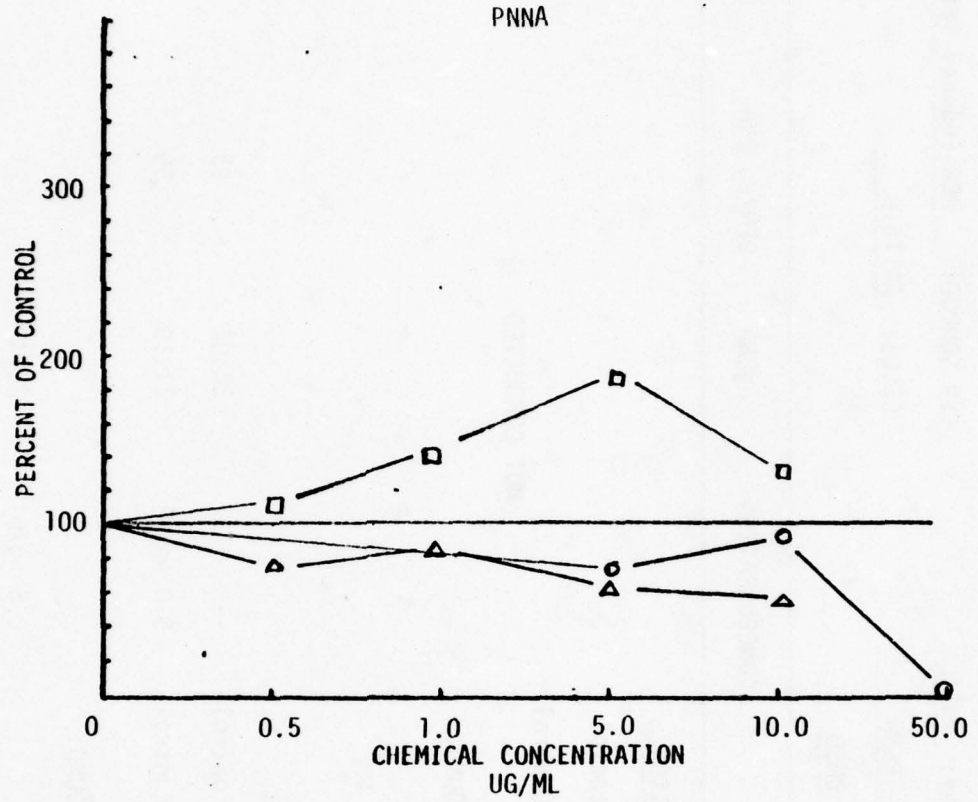
SOLVENT: DMSO

TEST	CONCENTRATION	µgDNA	DPM/µg DNA	PERCENT OF CONTROL
NONACTIVATION				
Solvent Control				
Positive Control (MNNG)	NOT CONDUCTED			
TEST COMPOUND:				
ACTIVATION				
Negative Control	--	22.04	25	100
Positive Control (BαP)	5.0 µg/ml	21.19	59	232
TEST COMPOUND:				
PNNA	0.5 µg/ml	22.47	27	107
PNNA	1.0 µg/ml	20.79	36	144
PNNA	5.0 µg/ml	17.00	47	189
PNNA	10.0 µg/ml	21.65	32	126

MNNG = N-methyl-N'-nitro-N-nitrosoguanidine
BαP = Benz( )pyrene

FIGURE 1
INCORPORATION OF ^{3}H-THYMIDINE
IN AG 1518 CELLS

PNNA



+S9 MIX DATA SHOWN

o Noninduced Mouse (Table 1)
△ Noninduced Mouse (Table 2)
□ PCB-induced Rat (Table 3)

PROTOCOL

1. PURPOSE

The purpose of this study was to evaluate the test material for its ability to induce unscheduled DNA synthesis (UDS) in normal human foreskin fibroblasts (obtained from the Institute for Medical Research, Camden, NJ (Repository No. AG 1518).

2. MATERIALS

A. Indicator Cells

Diploid normal human foreskin fibroblasts, Repository No. AG 1518, Passage 6.

B. Media

Growth medium (GM) consisted of Eagle's minimal essential medium (EMEM) supplemented with 10% fetal calf serum (FCS) and penicillin-streptomycin (PS).

Maintenance medium (SM) consisted of EMEM supplemented with 0.5% FCS and PS.

Hydroxyurea medium (HUM) consisted of SM plus hydroxyurea to a final concentration of 10^{-2} M.

C. Control Compounds

1. Negative control

The material used as the solvent for the test chemical was used as the negative control. The solvent is listed in Table 1 in Section V. Results. The volume of solvent in the negative control test was equal to the total solvent added in the high dose for the test chemical.

2. Positive controls

N-methyl nitrosoguanidine (MNNG) at a concentration of 5 µg/ml was used as the positive control agent in nonactivation tests, and Benzo(a)pyrene (BaP) at a concentration of 1 µg/ml to 10 ug/ml was used as the positive control agent in activation tests.

3. EXPERIMENTAL DESIGN

A. Cell Preparation

Normal human diploid foreskin fibroblasts were seeded at 5×10^5 cells in a 100 mm tissue culture dish and grown to confluency in GM. Once reaching confluency, the cells were switched to SM for 5 days. The contact inhibition imposed by confluency, and the use of SM, held the cells in a nonproliferating state.

B. Treatment

On the day of treatment, cells held in G_1 phase were placed in HUM. After 30 min, this medium was replaced by 2 ml HUM containing the control or test chemical, and 1.0 µCi ^{3}HTdR. An assay consisted of at least 3 concentrations. Exposure was terminated after 1.5 hr by washing the cells twice in a cold balanced salt solution (BSS) containing an excess of cold thymidine. The test concentrations were selected from a series of standard concentrations ranging from 0.1 µg/ml to 5.0 µg/ml. A lower series was used if all standard concentrations proved to be toxic.

Treated plates were frozen at -20°C until processed. After thawing, the cells on the 100 mm plate were covered with 0.1% sodium dodecyl sulfate (SDS) in (SSC) (0.15M sodium chloride-0.015M sodium citrate) and scraped from the dish with a rubber spatula. The cells were washed and precipitated from the SDS by 3 changes of 95% ethanol and centrifuged at 10,000 x g. Additional lipid components were removed by extraction in ethanol ether at 70°C. This pellet was washed in 70% ethanol, further incubated at 70°C in 0.3N NaOH, and the DNA extracted in 1000 µl 1N perchloric acid (PCA) at 70 C. The DNA was separated into two 500 µl aliquots. One of these was solubilized in 10 ml scintillation cocktail and counted in a Packard liquid scintillation spectrometer. The second aliquot was read at 260 nm in a UV spectrophotometer. The values were corrected for light scatter and converted to micrograms DNA. Following liquid scintillation counting, the data were combined with the DNA extraction values and expressed as disintegrations per minute per microgram DNA (DPM/µg DNA).

D. Activation System

Because metabolic activation is essential for the expression of biological activity in some chemicals, a liver activation system containing a noninduced mouse liver S9 and an Aroclor 1254 induced hepatic S9 fraction were employed. The activation system consisted of the following:

3. EXPERIMENTAL DESIGN (Continued)

D. Activation System

Component	Final Concentration/ml
NADP (sodium salt)	6 µmol
Isocitric acid	35 µmol
Tris buffer, pH 7.4	28 µmol
$MgCl_2$	2 µmol
Liver S9 (9,000 x g)	100 µl

4. EVALUATION CRITERIA

Several criteria have been established which, if met, provide a basis for declaring a material genetically active in the UDS assay. These criteria are derived from a historical data base and are helpful in maintaining uniformity in evaluations from material to material and run to run. While these criteria are reasonably objective, a certain amount of flexibility may be required in making the final evaluations since absolute criteria may not be applicable to all biological data.

A compound is considered active in the UDS assay if:

a. A dose-response relationship is observed over two consecutive dose levels.

b. The minimum increase at the high level of the dose response is approximately 150% of the control value. The positive control data for a large sampling 5 µg/ml of MNNG tests was found to be 206% of the control and for 10 µg/ml of Benz(a)pyrene it was 162% of the control.

All evaluations of UDS activity are based on the concurrent solvent control value run with the experiment in question. Positive control values are not used as reference points to measure activity, but rather to demonstrate that the cell population employed was responsive to chemicals known to induce repair synthesis under the appropriate test conditions.

As the data base for the UDS assay increases, the evaluation criteria will be more firmly established.

MUTAGENICITY EVALUATION OF

1-P-NITROPHENYLAZO-2-NAPHTHALENEAMINE (PNNA)

IN THE MOUSE DOMINANT LETHAL ASSAY

FINAL REPORT

DECEMBER 1978

I. SPONSOR: United States Army

II. MATERIAL*

A. Identification: PNNA

B. Date Received: October 18, 1977

C. Physical Description: Dark red powder

III. TYPE OF ASSAY: Mouse Dominant Lethal Assay

IV. PROTOCOL NO.: DMT-110

V. RESULTS

The results are presented in Tables 2-7. Table 1 provides information on toxicity and dose selection. The remaining tables summarize the test results and statistical analyses.

VI. INTERPRETATION OF RESULTS AND CONCLUSIONS

A series of concentrations employing the test material at 0.05 g/kg to 5.0 g/kg were used in the preliminary toxicity test. No significant effects were observed after 14 days, and the 5.0 g/kg was selected as a high dose. This was based on the determination of nontoxicity and using 5.0 g/kg as the highest concentration of a nontoxic material.

The data presented in Tables 2-7 summarize the results of the dominant lethal study. All of the data were considered negative. The only increased parameters were observed at the two low doses at Week 3 on Table 6. These increases appeared to result from a low number of pregnant females at the third week. All other parameters did not show any increases. TEM was used as a positive control in this assay and produced the expected effects over Weeks 1-3.

*Information was supplied by the sponsor. If the sponsor did not indicate this information, N.I. was entered.

VI. INTERPRETATION OF RESULTS AND CONCLUSIONS (Continued)

The test material, PNNA was not active in the Mouse Dominant Lethal Assay conducted as part of this evaluation.

Submitted by:

Study Director

Dale W. Matheson, Ph.D. — Date 4/27/78
Associate Director and
Section Chief
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Reviewed by:

David Brusick, Ph.D. — Date 4/27/78
Director
Department of Genetics
and Cell Biology

TABLE 1

COMPOUND: PNNA

ASSAY#: 2414

CLIENT: U.S. Army

PROJECT#: 20881

TEST INITIATED: 12/22/77

BY: A.T.

SOLVENT: DMSO

SPECIES/PO#: CD-1 Male Mice #78637

ROUTE OF ADMINISTRATION: Oral

DOSE UNITS: g/kg

DOSING INFORMATION

Treatment	Compound	Route of Administration
0.05 g/kg	PNNA	P.O.
0.1 g/kg	PNNA	P.O.
0.5 g/kg	PNNA	P.O.
1.0 g/kg	PNNA	P.O.
5.0 g/kg	PNNA	P.O.

TABLE 2

FERTILITY INDEX

COMPOUND: PNNA STUDY: SUBCHRONIC SPECIES: MICE

WEEK	HIST. CONTROL	NEG. CONTROL	POS. CONTROL	0.500 GM/KG	1.000 GM/KG	5.000 GM/KG		LOG DOSE	ARITH DOSE
1	407/ 740 = 0.55	6/ 20 = 0.30	4/ 20 = 0.20	11/ 20 = 0.55	9/ 20 = 0.45	5/ 20 = 0.25	I		
2	515/ 740 = 0.70	12/ 20 = 0.60	3/ 20 = 0.15**	18/ 20 = 0.90	14/ 20 = 0.70	9/ 20 = 0.45	I	$$	$
3	506/ 740 = 0.68	15/ 20 = 0.75	16/ 20 = 0.80	12/ 20 = 0.60	14/ 20 = 0.70	12/ 20 = 0.60	I		
4	508/ 740 = 0.69	16/ 20 = 0.80	16/ 20 = 0.80	15/ 20 = 0.75	9/ 20 = 0.45*	11/ 20 = 0.55	I		
5	479/ 739 = 0.65	14/ 20 = 0.70	17/ 20 = 0.85	17/ 20 = 0.85	15/ 20 = 0.75	15/ 20 = 0.75	I		
6	499/ 740 = 0.67	16/ 20 = 0.80	17/ 20 = 0.85	20/ 20 = 1.00	16/ 20 = 0.80	15/ 20 = 0.75	I		
7	492/ 740 = 0.66	16/ 20 = 0.80	17/ 20 = 0.85	15/ 20 = 0.75	14/ 20 = 0.70	10/ 20 = 0.50	I		$

NOTE: THE SYMBOLS $ AND * DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL OR HISTORICAL CONTROL GROUP.

* INDICATES SIGNIFICANT DIFFERENCE FROM CONTROL.
$ INDICATES SIGNIFICANT LINEAR RELATIONSHIP WITH ARITH OR LOG DOSE.

ONE $ OR * INDICATES SIGNIFICANCE AT P LESS THAN 0.05.
TWO $ OR * INDICATES SIGNIFICANCE AT P LESS THAN 0.01.

TABLE 3

AVERAGE NUMBER OF IMPLANTATIONS PER PREGNANT FEMALE

COMPOUND: PNNA STUDY: SUBCHRONIC SPECIES: MICE

WEEK	HIST. CONTROL	NEG. CONTROL	POS. CONTROL	0.500 GM/KG	1.000 GM/KG	5.000 GM/KG	LOG DOSE	ARITH DOSE
1	4695/ 407 = 11.5	59/ 6 = 9.8	26/ 4 = 6.5**	114/ 11 = 10.4	95/ 9 = 10.6	46/ 5 = 9.2	$	$
2	6036/ 515 = 11.7	124/ 12 = 10.3	17/ 3 = 5.7*	211/ 18 = 11.7	165/ 14 = 11.8	114/ 9 = 12.7		
3	5972/ 506 = 11.8	168/ 15 = 11.2	132/ 16 = 8.3**	146/ 12 = 12.2	156/ 14 = 11.1	140/ 12 = 11.7		
4	5927/ 508 = 11.7	207/ 16 = 12.9	173/ 16 = 10.8*	185/ 15 = 12.3	105/ 9 = 11.7	131/ 11 = 11.9		
5	5537/ 479 = 11.6	159/ 14 = 11.4	187/ 17 = 11.0	205/ 17 = 12.1	178/ 15 = 11.9	175/ 15 = 11.7		
6	6018/ 499 = 12.1	163/ 16 = 10.2	188/ 17 = 11.1	226/ 20 = 11.3*	170/ 16 = 10.6	162/ 15 = 10.8		
7	5952/ 492 = 12.1	191/ 16 = 11.9	206/ 17 = 12.1	167/ 15 = 11.1	149/ 14 = 10.6	124/ 10 = 12.4		

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TWO $ OR * INDICATES SIGNIFICANCE AT P LESS THAN 0.01.

TABLE 4

AVERAGE RESORPTIONS (DEAD IMPLANTS) PER PREGNANT FEMALE
COMPOUND: PNNA STUDY: SUBCHRONIC SPECIES: MICE

WEEK	HIST. CONTROL	NEG. CONTROL	POS. CONTROL	0.500 GM/KG	1.000 GM/KG	5.000 GM/KG	LOG DOSE	ARITH DOSE
1	261/ 407 = 0.64	7/ 6 = 1.17	18/ 4 = 4.50**	6/ 11 = 0.55	7/ 9 = 0.78	0/ 5 = 0.0		$
2	450/ 515 = 0.87	5/ 12 = 0.42	13/ 3 = 4.33**	24/ 18 = 1.33	17/ 14 = 1.21	6/ 9 = 0.67		
3	388/ 506 = 0.77	11/ 15 = 0.73	55/ 16 = 3.44**	23/ 12 = 1.92	28/ 14 = 2.00*	10/ 12 = 0.83		
4	418/ 508 = 0.82	8/ 16 = 0.50	12/ 16 = 0.75	12/ 15 = 0.80	4/ 9 = 0.44	14/ 11 = 1.27		
5	287/ 479 = 0.60	7/ 14 = 0.50	10/ 17 = 0.59	12/ 17 = 0.71	9/ 15 = 0.60	11/ 15 = 0.73		
6	369/ 499 = 0.74	6/ 16 = 0.38	17/ 17 = 1.00	9/ 20 = 0.45	14/ 16 = 0.88	19/ 15 = 1.27		
7	305/ 492 = 0.62	9/ 16 = 0.56	12/ 17 = 0.71	6/ 15 = 0.40	9/ 14 = 0.64	10/ 10 = 1.00		

NOTE: THE SYMBOLS $ AND * DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL OR HISTORICAL CONTROL GROUP.

* INDICATES SIGNIFICANT DIFFERENCE FROM CONTROL.
$ INDICATES SIGNIFICANT LINEAR RELATIONSHIP WITH ARITH OR LOG DOSE.

ONE $ OR * INDICATES SIGNIFICANCE AT P LESS THAN 0.05.
TWO $ OR * INDICATES SIGNIFICANCE AT P LESS THAN 0.01.

TABLE 5

PROPORTION OF FEMALES WITH ONE OR MORE DEAD IMPLANTATIONS
COMPOUND: PNNA STUDY: SUBCHRONIC SPECIES: MICE

WEEK	HIST. CONTROL	NEG. CONTROL	POS. CONTROL	0.500 GM/KG	1.000 GM/KG	5.000 GM/KG	LOG DOSE	ARITH DOSE
1	173/ 407 = 0.43	3/ 6 = 0.50	4/ 4 = 1.00	6/ 11 = 0.55	6/ 9 = 0.67	0/ 5 = 0.0		$
2	193/ 515 = 0.37	4/ 12 = 0.33	3/ 3 = 1.00	10/ 18 = 0.56	10/ 14 = 0.71	5/ 9 = 0.56		
3	229/ 506 = 0.45	7/ 15 = 0.47	16/ 16 = 1.00**	8/ 12 = 0.67	10/ 14 = 0.71	7/ 12 = 0.58		
4	255/ 508 = 0.50	7/ 16 = 0.44	9/ 16 = 0.56	7/ 15 = 0.47	3/ 9 = 0.33	7/ 11 = 0.64		
5	183/ 479 = 0.38	5/ 14 = 0.36	10/ 17 = 0.59	9/ 17 = 0.53	7/ 15 = 0.47	8/ 15 = 0.53		
6	220/ 499 = 0.44	5/ 16 = 0.31	9/ 17 = 0.53	7/ 20 = 0.35	6/ 16 = 0.38	8/ 15 = 0.53		
7	214/ 492 = 0.43	6/ 16 = 0.38	7/ 17 = 0.41	4/ 15 = 0.27	5/ 14 = 0.36	5/ 10 = 0.50		

NOTE: THE SYMBOLS $ AND * DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL OR HISTORICAL CONTROL GROUP.

* INDICATES SIGNIFICANT DIFFERENCE FROM CONTROL.
$ INDICATES SIGNIFICANT LINEAR RELATIONSHIP WITH ARITH OR LOG DOSE.

ONE $ OR * INDICATES SIGNIFICANCE AT P LESS THAN 0.05.
TWO $ OR * INDICATES SIGNIFICANCE AT P LESS THAN 0.01.

TABLE 6

PROPORTION OF FEMALES WITH TWO OR MORE DEAD IMPLANTATIONS
COMPOUND: PNNA STUDY: SUBCHRONIC SPECIES: MICE

WEEK	HIST. CONTROL	NEG. CONTROL	POS. CONTROL	0.500 GM/KG	1.000 GM/KG	5.000 GM/KG	LOG DOSE	ARITH DOSE
1	67/ 407 = 0.16	2/ 6 = 0.33	4/ 4 = 1.00	0/ 11 = 0.0	1/ 9 = 0.11	0/ 5 = 0.0		
2	94/ 515 = 0.18	1/ 12 = 0.08	3/ 3 = 1.00*	3/ 18 = 0.17	4/ 14 = 0.29	1/ 9 = 0.11		
3	76/ 506 = 0.15	2/ 15 = 0.13	15/ 16 = 0.94**	7/ 12 = 0.58*	7/ 14 = 0.50	2/ 12 = 0.17	$	
4	92/ 508 = 0.18	1/ 16 = 0.06	3/ 16 = 0.19	4/ 15 = 0.27	1/ 9 = 0.11	3/ 11 = 0.27		
5	61/ 479 = 0.13	2/ 14 = 0.14	0/ 17 = 0.0	3/ 17 = 0.18	2/ 15 = 0.13	1/ 15 = 0.07		
6	73/ 499 = 0.15	1/ 16 = 0.06	4/ 17 = 0.24	2/ 20 = 0.10	2/ 16 = 0.13	4/ 15 = 0.27		
7	59/ 492 = 0.12	2/ 16 = 0.13	3/ 17 = 0.18	1/ 15 = 0.07	2/ 14 = 0.14	1/ 10 = 0.10		

NOTE: THE SYMBOLS $ AND * DENOTE SIGNIFICANT RELATIONSHIPS AND DIFFERENCES USING THE NEGATIVE CONTROL OR HISTORICAL CONTROL GROUP.

* INDICATES SIGNIFICANT DIFFERENCE FROM CONTROL.
$ INDICATES SIGNIFICANT LINEAR RELATIONSHIP WITH ARITH OR LOG DOSE.

ONE $ OR * INDICATES SIGNIFICANCE AT P LESS THAN 0.05.
TWO $ OR * INDICATES SIGNIFICANCE AT P LESS THAN 0.01.

TABLE 7

DEAD IMPLANTS / TOTAL IMPLANTS

COMPOUND: PNNA STUDY: SUBCHRONIC SPECIES: MICE

WEEK	HIST. CONTROL	NEG. CONTROL	POS. CONTROL	0.500 GM/KG	1.000 GM/KG	5.000 GM/KG	
1	261/4695 = 0.06	7/ 59 = 0.12	18/ 26 = 0.69**	6/114 = 0.05	7/ 95 = 0.07	0/ 46 = 0.0	I
2	450/6036 = 0.07	5/124 = 0.04	13/ 17 = 0.76**	24/211 = 0.11	17/165 = 0.10	6/114 = 0.05	I
3	388/5972 = 0.06	11/168 = 0.07	55/132 = 0.42**	23/146 = 0.16	28/156 = 0.18*	10/140 = 0.07	I
4	418/5927 = 0.07	8/207 = 0.04	12/173 = 0.07	12/185 = 0.06	4/105 = 0.04	14/131 = 0.11	I
5	287/5537 = 0.05	7/159 = 0.04	10/187 = 0.05	12/205 = 0.06	9/178 = 0.05	11/175 = 0.06	I
6	369/6018 = 0.06	6/163 = 0.04	17/188 = 0.09	9/226 = 0.04	14/170 = 0.08	19/162 = 0.12	I
7	305/5952 = 0.05	9/191 = 0.05	12/206 = 0.06	6/167 = 0.04	9/149 = 0.06	10/124 = 0.08	I

NOTE: THE SYMBOL * DENOTES SIGNIFICANT DIFFERENCE USING THE NEGATIVE CONTROL GROUP.
ONE * INDICATES SIGNIFICANCE AT P LESS THAN 0.05.
TWO * INDICATES SIGNIFICANCE AT P LESS THAN 0.01.

PROTOCOL

1. PURPOSE

The purpose of this study was to evaluate the test material for its ability to induce dominant lethality in mice.

2. OVERVIEW AND RATIONALE

The dominant lethal assay is designed to determine the ability of a compound to induce genetic damage in the germ cells of treated male mice leading to fetal wastage. Chromosome aberrations including breaks, rearrangements, and deletions are believed to produce the dominant lethality although ploidy changes and chromosome nondisjunction may also be detected in this assay. Male mice are exposed to several dose levels of the test compound for 5 days and then mated over the entire period of spermatogenesis to unexposed virgin females. At mid-pregnancy, the females are killed and scored for the number of living and dead implants as well as the level of fertility. These results are then compared to data from control animals and used to determine the degree of induced dominant lethality.

Evidence of dominant lethality emphasizes that the compound is able to reach the developing germ cells and induce genetic damage. It also suggests, but does not measure directly, that in addition to the detected gross chromosomal lesions, more subtle balanced lesions or specific locus gene mutations may be produced. These latter types have a good chance of being transmitted to the gene pool of future offspring.

3. EXPERIMENTAL DESIGN

Ten (10) random bred, male mice from a closed colony were assigned to 1 of 5 groups. Three of these groups received different dose levels of the test compound; a fourth group received only the solvent; and the fifth group received a known mutagen and served as the positive control group. The test compound and the solvent control were administered orally for 5 consecutive days. Triethylenemelamine (TEM) was used as the positive control and was given as a single intraperitoneal injection 2 days before the animals were mated. Following treatment, each male was rested for 2 days and then caged with 2 unexposed virgin females. At the end of 7 days, these females were replaced with 2 new unexposed females. This weekly mating sequence was continued for 7 weeks. The 2 mated females were transferred to a new cage, and 14 days after the midweek of being caged with the male, the females were killed with CO_2. At necropsy, their uteri were examined for dead and living fetuses, resorption sites, and total implantations. Animals which died during dosing were not replaced unless there was 75% mortality at a single dose level. In that case the compound toxicity was reviewed, and the entire dose level was repeated.

3 EXPERIMENTAL DESIGN (Continued)

A. Animals

Random bred, male and female mice, strain CD-1, were purchased from the Charles River Breeding Laboratories, Inc., Portage, Michigan. Male and female mice were at least 8 weeks of age when purchased.

B. Animal Husbandry

Males were housed individually and females housed in pairs (except during mating) in shoe box cages on AB-SORB-DRI bedding.

All animals were quarantined for 2 weeks prior to being used in the study to acclimate them to the new laboratory conditions. Purina Lab Chow was used as the basic diet and water was offered ad libitum. Light was provided on a 12-hour light/dark cycle.

Personnel handling animals or working within the animal facility wore suitable protective laboratory garments, including face masks or respirators.

C. Dosage Determination

Dosage information was calculated on the basis of range finding studies using groups of 6 mice each. The high dose level was selected from these data. One-third and one-tenth of the high dose were used as the intermediate and low dose levels, respectively. Nontoxic compounds are tested at 5.0 g/kg as the high dose.

D. Records

The number of dead and living fetuses, resorption sites, and total implantation sites were recorded on a standardized record form. Data were keypunched directly from these forms to computer entry cards, and analyzed for statistical significance as outlined in Appendix A. Original copies of all data are stored in the Litton Bionetics, Inc. archival system.

4. EVALUATION CRITERIA

Both pre- and post-implantation losses contribute to dominant lethality. The former is reflected in the total number of implantation sites per pregnant female and strictly measured by the difference between the number of corpora lutea gravidus and the number of implantation sites. Toxic or physiological effects on sperm may also reduce the number of implantation sites. Therefore, unless subtle physiological effects on sperm can be discounted, pre-implantation loss is not as rigorous an indication of dominant lethality as post-implantation loss. Corpora lutea cannot be reliably counted in mice and, therefore, pre-implantation loss is not evaluated in studies using mice. Post-implantation losses are measured as early and late fetal deaths plus the number of resorption sites.

Dominant lethality is typically determined from: a) a mutation index derived from the ratio of dead to total implants; or b) the number of dead implants per pregnant female. In interpreting these values it must be remembered that the former measurement reflects both pre- and post-implantation losses and that the ratio is affected by changes in either the numerator or the denominator. For this reason the second parameter is perhaps a better indicator of post-implantation loss. This becomes especially so if one concurrently examines the number of living embryos per pregnant female. The two sets of data should be inversely related. In other words, if true dominant lethality is being observed, then a significant increase in the number of dead implants per pregnant female should be accompanied by a significant decrease in the number of living implants per pregnant female.

These ratios are compared with both concurrent and historical control data for significant statistical differences. Dose-related trends are also looked for, but may not always be found. For example, some compounds such as EMS tested in mice show a threshold value and then a very steep rise. Certain portions of the response might be missed, depending on the spacing of the dose levels used.

True, as opposed to spurious, dominant lethality also tends to cluster according to the stage of spermatogenesis affected and typically would not be expected to appear in widely spaced weeks or blocks of weeks.

All data which are indicated as being statistically significant must also be strongly evaluated for their biological significance. By bringing both statistical and biological selective pressures to bear on the data gathered, an estimate of dominant lethality and of risk to the gene pool should be obtainable.

5. STANDARD OPERATING PROCEDURE

The test compound will be registered in a bound log book recording the date of receipt, complete client identification, physical description, and Litton Bionetics, Inc. code number.

Complete records of weights and dilutions associated with the testing of the submitted material will be entered into a bound notebook.

Raw data information will be recorded on special printed forms that will be dated and initialed by the individual performing the data collection at the time the observations are made. These forms will be filed as permanent records.

All data will be entered in ink (no pencil).

All changes or corrections in entries will be made with a single line through the change, and an explanation for the change must be written.

All calculations (weights, dilutions, dose calculations, etc.) will be shown on data records.

All data entries will be dated and initialed.

All laboratory operations will be written out in standard protocol manuals. These manuals will be present in each laboratory area.

Deviations from any established protocol will be described and justified.

Data will be stored in bound form (notebooks or binders). These bound data books will be reviewed by the appropriate Section Heads.

Chemicals submitted for testing will have date of receipt and initials of entering person.

Lot numbers for all reference mutagens, solvent, or other materials used in assays will be recorded.

Animal orders, receipts, and identification will be recorded and maintained such that each animal can be traced to the supplier and shipment. All animals on study will be properly identified.

A copy of the final report plus all raw data and support documents will be permanently stored in the archival system of Litton Bionetics, Inc.

Current curricula vitae and job descriptions will be maintained on all personnel involved in the study.

REFERENCES

Epstein, S.S., Arnold, E., Andrea, J., Bass, W., and Bishop, Y. (1972). Detection of chemical mutagens by the dominant lethal assay in the mouse. Toxicol. Appl. Pharmacol. 23, 288-325.

Ray, V.A. and Hyneck, M.L. (1973). Some primary considerations in the interpretation of the dominant-lethal assay. Environmental Health Perspectives 6, 27.

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APPENDIX A

DESCRIPTION OF STATISTICAL ANALYSIS USED FOR THE DOMINANT LETHAL STUDY

APPENDIX A

Analysis of Data

1. Fertility Index - Table 1

a. The fertility index is defined as F.I. = # of pregnant females/ # of mated females. It is calculated for each week (in subacute study) or at the end of 8 weeks (in acute study) and for each dose level, negative control, and positive control.

b. A chi-square test is used to compare each treatment group and positive control to negative control.

$$\chi_i^2 = \frac{(N_0 + N_i)\,(|n_0(N_i - n_i) - n_i(N_0 - n_0)| - (N_0 + N_i)/2)^2}{(n_0 + n_i)(N_0 - n_0 + N_i - n_i)N_0N_i}$$

where

n_i = # impregnated in i-th test group

n_0 = # impregnated in negative control group

N_i = # of females mated in the i-th test group

N_0 = # of females mated in negative control group

A 2 x 2 table is formed as follows:

	control	test
# impreg	n_0	n_i
# not impreg	$N_0 - n_0$	$N_i - n_i$

Significance at the 5 and 1% levels is indicated with asterisks on Table 1.

c. Armitage's trend for linear proportions is used to test whether the fertility index is linearly related to arithmetic or log dose.

The following table is set up:

	-control	dose 1	dose 2	dose 3	dose k	totals
# impreg	n_0	n_1	n_2	n_3	n_k	t
# not impreg	$N_0 - n_0$	$N_1 - n_1$	$N_2 - n_2$	$N_3 - n_3$	$N_k - n_k$	T - t
totals	N_0	N_1	N_2	N_3	N_k	T

and Armitage's chi-square is calculated:

$$\chi_A^2 = \chi_{(k-1)}^2 - \chi_1^2$$

where

$$\chi_1^2 = \frac{T(\sum_{i=0}^{k} n_i x_i - t\sum_{i=0}^{k} N_i x_i)^2}{t(T - t)(\sum_{i=0}^{k} N_i x_i^2 - (\sum_{i=0}^{k} N_i x_i)^2)}$$

$$\chi_{(k-1)}^2 = \frac{T^2(\sum_{i=0}^{k} n_i^2/N_i - t^2/T)}{t(T - t)}$$

and the x_i are the dose levels. This calculation is repeated with x replaced by $\log_{10} x$. The 5 and 1% significance levels are indicated by dollar signs on Table 1.

2. Total Number of Implantations - Tables 2, 2A

a. The total number of implantations is evaluated by the Student's t-test to determine whether the average number of implantations per pregnant female for each treatment group and the positive control group differs significantly from the negative control group.

n_i = # of pregnant females at dose level i.
u_{ij} = # of implantations for pregnant female j in dose group i.

$$\bar{u}_i = 1/n_i(\sum_{j=1}^{n_i} u_{ij})$$

$$s_i^2 = \sum_{j=1}^{n_i} (u_{ij} - \bar{u}_i)^2$$

$$t_i = |\bar{u}_0 - \bar{u}_i| / \left(\frac{s_0^2 + s_i^2}{n_0 + n_i - 2} \left(\frac{1}{n_0} + \frac{1}{n_i}\right)\right)^{\frac{1}{2}}$$

$$d.f. = n_0 + n_i - 2$$

Significance at the 5 and 1% levels is indicated by asterisks in Table 2.

b. A regression fit of the average number of implantations, $\bar{u}_i$, is made for both the arithmetic and logarithmic dose (x_i and log x_i). The doses x_i are used as independent variables and the fit includes data from the three treatment groups and the control group.

N = total # of pregnant females in all groups.

x_i = dose/log (dose) for the i-th female.

U_i = # of implantations for the i-th female.

$$\bar{x} = \frac{1}{N}\sum_{i=1}^{N} x_i$$

$$SS_x = \sum_{i=1}^{N} (x_i - \bar{x})^2$$

$$\bar{U} = \frac{1}{N}\sum_{i=1}^{N} U_i$$

$$SS_u = \sum_{i=1}^{N} (U_i - \bar{U})^2$$

$$S_{xu} = \sum_{i=1}^{N} (x_i - \bar{x})(U_i - \bar{U})$$

B = estimate of slope of regression line = S_{xu}/SS_x

A = estimate of intercept of regression line = $\bar{U} - B\,\bar{x}$

VARU = variance of U about regression line

$$= \frac{SS_u - S_{xu}2/SS_x}{N-2}$$

VARB = variance of B = $\frac{VARU}{SS_x}$

VARA = variance of A = $VARU\ (\frac{1}{N} + \frac{\bar{x}^2}{SS_x})$

TB = $B/(VARB)^{\frac{1}{2}}$ = t-statistic for testing the hypothesis that the regression slope is zero

DF = N-2 = # of degrees of freedom for T B

CVUX = coefficient of variation of U about x

= $(VARU.X)^{\frac{1}{2}}/\bar{U}$

VARU.X = $\frac{1}{N-2}\ (SS_U - S_{XU}^2/SS_X)$

SDY = standard deviation of U about the regression line

= $(VARU.X)^{\frac{1}{2}}$

SDS = standard deviation of the slope

= $(VARB)^{\frac{1}{2}}$

SDA = standard deviation of intercept

= $(VARA)^{\frac{1}{2}}$

Significant difference of the slope from zero is indicated at the 5 and 1% levels in Table 2. Table 2A shows detailed results of the regression analysis.

3. Total Number of Corpora Lutea - Tables 3, 3A
(For rats only)

a. The average number of corpora lutea per pregnant female is evaluated by t-test to determine whether each treatment group differed significantly from the control group. Use the equation described in Step 2 above with

u_{ij} = # of corpora lutea for pregnant female j in dose group i.

b. A regression fit of the average number of corpora lutea per pregnant female is made for both the arithmetic and logarithmic dose. Use the equations described in Step 2 above with

u_i = # of corpora lutea for the i-th female

4. Preimplantation Losses - Tables 4, 4A
(For rats only)

a. The number of preimplantation losses is the number of corpora lutea minus the number of implantations.

Y_{ij} = preimplantation losses for j-th female in i-th group

V_{ij} = # of corpora lutea for j-th female in the i-th group

b. The Freeman-Tukey transformation is applied to the Y_{ij} as follows:

$$f_{ij} = \sin^{-1}\sqrt{\frac{y_{ij}}{V_{ij}+1}} + \sin^{-1}\sqrt{\frac{y_{ij}+1}{V_{ij}+1}}$$

The t-test is then applied to the f's, comparing the test groups to the negative control. Let

$$\bar{f}_i = \frac{1}{n_i}\sum_{j=1}^{n_i} f_{ij}$$

$$s_i^2 = \sum_{j=1}^{n_i} (f_{ij} - \bar{f}_i)^2$$

where n_i = # of pregnant females at dose level i.

$$\text{Then } t = (\bar{f}_0 - \bar{f}_i)/\left[\frac{s_0^2 + s_i^2}{n_0 + n_i - 2}\left(\frac{1}{n_0} + \frac{1}{n_i}\right)\right]^{\frac{1}{2}}$$

c. Regression analysis is used to determine whether the average number of preimplantation losses per female is related to the arithmetic or the log dose. The method is as used in Step 2 above substituting

U_i = # of preimplantation losses for the i-th female.

5. Dead Implantations - Tables 5, 5A

The dead implants were evaluated by the same statistical techniques that were used in evaluating the total number of implantations.

Substitute

u_{ij} = # of dead implants for j-th female in the i-th group in the equations in Step 2 above.

6. Proportion of Females with One or More Dead Implantations - Table 6

The proportion of females with one or more dead implants is the number of females with dead implants/number of pregnant females. These proportions are analyzed by the same method used to analyze the fertility indices, i.e., by a chi-square test and Armitage's trend.

Substitute n_i = # of pregnant females with one or more dead implants at dose level i and

N_i = # of pregnant females at dose level i in Step 1 above.

Also a probit regression analysis is done using these proportions, p_i, to determine whether the probit of p_i is linearly related to the log or arithmetic dose. The Biomedical Computer Program BMD03S is used to compute A and B and the χ^2 statistic for the regression equations $y = A + B\,x$ and $y = A + B \log x$.

7. Proportion of Females with Two or More Dead Implantations - Table 7

The proportion of females with two or more dead implantations is the number of females with two or more dead implants/number of pregnant females. The data are evaluated by the same method used for evaluating the proportion of females with one or more dead implants.

8. Dead Implants/Total Implants - Table 8

Dead implants/total implants were computed for each female and transformed by way of the Freeman-Tukey arc-sine transformation prior to being evaluated by t-test to compare each treatment group and positive control to negative control.

Use y_{ij} = # dead implants for j-th female in i-th group

v_{ij} = # of total implants for j-th female in i-th group

in the equations in Step 4 above.

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